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HEPATOPROTECTIVE EFFECT OF *FLACOURTIA JANGOMAS* (LOUR.) RAEUSCH ON PARACETAMOL-INDUCED LIVER INJURY IN RATS

by

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INTRODUCTION AND REVIEW OF LITERATURE

The liver is the core organ concerned with the synthesis, excretion, metabolism, and detoxification of varying exogenous and endogenous substances. Sustained exposure of the liver to these toxic substances can impose liver damage (Saidurrahman *et al*., 2022). Liver diseases have become a global concern worldwide. A survey report by the World Health Organization projects that more than 10 % of the world's population is suffering from liver injury (Ren *et al*., 2019). Every year deaths caused by liver diseases are rising at an appalling rate. It has been increasing over the years and the World Health Organisation (WHO) has forecasted in its World Health Statistics of 2020 to be the eleventh most important cause of death in the world by 2030 (Meshack and Gupta, 2022). Liver disease has been the tenth most common cause of death in India in 2020 (Meshack and Gupta, 2022).

Liver damage can be induced by various factors: bacteria, viruses, parasites, drugs, autoimmune diseases, toxic compounds, and excessive consumption of alcohol (Madrigal-Santillán *et al*., 2014). Among all these agents, medications are the most common contributing agent for liver diseases. Hepatotoxicity induced by the drug is a well-known recognized problem worldwide (Gebremedhin *et al*., 2020).

Acetaminophen (paracetamol (PCM) or N-acetyl-para-aminophenol (APAP)) is an over the counterdrug used as an analgesic and antipyretic worldwide (Mazaleuskaya *et al*., 2015). At therapeutic doses, paracetamol is metabolized in the liver through glucuronidation, and sulphation, with less than 5-10 % oxidized by cytochrome p450 isozymes to N-acetyl-p-benzoquinone imine (NAPQI) (Kalsi *et al*., 2011). NAPQI, produced during the oxidation of APAP will react with glutathione (GSH) in the liver cell and excreted through the kidney (Sinaga *et al*., 2021). At overdose of paracetamol, glucuronidation/sulfation conjugation pathways are saturated, and NAPQI is produced (Kalsi *et al*., 2011). Due to the depletion in glutathione, NAPQI accumulates and oxidizes macromolecules in liver tissue such as lipid or -SH group of protein causing oxidative stress and hepatic necrosis (Gebremedhin *et al*., 2020).

Liver illnesses are of major concern, and standard medications available for the treatment are sometimes ineffective, expensive, and can have dangerous side effects. Hence, there is an increased interest in medicinal plants as potential sources of hepatoprotective compounds. A varied range of plant-based traditional remedies or formulations comprising herbal extracts is available on the market for the treatment of liver diseases. Phytonutrients,

coumarins, lignans, essential oils, monoterpenes, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids, and xanthines are among the chemical elements found in hepatoprotective herbal medications (Kumar *et al*., 2022). The WHO estimates that 80 % of the populations of some Asian and African countries presently use herbal medications for some aspect of primary health care (Oyebode *et al*., 2016). Silymarin and resveratrol are two of many examples of natural substances that have shown a strong hepatoprotective potential due to their antioxidant, anti-inflammatory, and liver regenerative capabilities (Farghali *et al*., 2015).

Flacourtia jangomas (Lour.) Raeusch is one among those plants that have not been adequately explored scientifically. *F. jangomas* commonly recognized as the Indian coffee plum is an exotic, semi-wild, tropical, and indigenous medicinal fruit that belongs to the Salicaceae family and is grown widely in the South-eastern and Eastern parts of Asia predominately in the North-eastern states of India (Barbhuiya *et al*., 2020). Traditionally, fruits of *F. jangomas* are used in biliousness, fever, and digestive disorders. Leaves and barks are used in the treatment of diarrhoea, bleeding gums, toothache, piles, and weakness of limbs. The roots are sweet, refrigerant, depurative, alexipharmic and diuretic. They are useful in asthma, and anaemia. The leaves and young shoots are astringent and stomachic. Barks are used for the treatment of intermittent fever (Sasi *et al*., 2018). Flacourtiaceae elaborates a diverse array of compound classes which include terpenoids, alkaloids, flavonoids and tannins, lignans and flavonolignans, glucosides, coumarins, isocoumarins (Rai and Mishra, 2020).

However, there is no scientific basis for *F. jangomas* as a hepatoprotective agent. The present study was carried out to evaluate the hepatoprotective activity of *F. jangomas* on paracetamol-induced liver injury.

SCOPE OF THE STUDY

As the liver is the central organ of metabolism, it is a highly vulnerable target for injury from drugs and chemicals. Paracetamol was first introduced in the year 1955 for its clinical application and since then, it has been widely used almost throughout the world. In many countries, the drug is readily available over the counter. Its easy availability without the need for a prescription made it one of the most common drugs used as an analgesic and antipyretic drug. Due to its easy availability in the market, lay people commonly

underestimate its toxicity. Paracetamol toxicity is one of the most common causes of poisoning worldwide. Currently, paracetamol is the most common cause of acute liver failure. Studies suggest that pathologic evaluation of various organs shows that the liver is a primary target for toxicity after paracetamol overdose because the hepatocytes elaborate NAPQI. As NAPQI has a short life span, it can damage only cells that elaborate it. Overdose of paracetamol may produce severe liver injury with hepatocellular necrosis. Even though various treatments are available, people tend to prefer complementary medicine such as herbal remedies instead of allopathic medicine. Plants act as a source of very effective remedies for a good number of human health hazards. The medical effectiveness of plants is due to the presence of a wide array of bioactive phytoconstituents that record significant antioxidant activity. *F. jangomas* is one of the underutilized fruit plants in India. Hence, the present study was carried out to explore the hepatoprotective activity of *F. jangomas* leaf and fruit methanolic extract on paracetamol-induced liver injury.

OBJECTIVES

1. To evaluate the physicochemical properties and phytochemicals of *Flacourtia jangomas* (Lour.) Raeusch leaf and fruit.
2. To evaluate the safety profile of methanolic extract from the leaf and fruit of *F. jangomas* (Lour.) Raeusch.
3. To evaluate the hepatoprotective activity of *F. jangomas* (Lour.) Raeusch leaf and fruit methanolic extract.
4. To identify the bioactive constituents by GC-MS analysis and to elucidate by molecular docking the mechanisms underlying the hepatoprotective activity of *F. jangomas* (Lour.) Raeusch leaf and fruit methanolic extract.

GENERAL MATERIALS AND METHODS

Collection of plant material:

Flacourtia jangomas (Lour.) Raeusch was collected from Bellikoth, Kasargod District of Kerala, India. It was taxonomically identified and authenticated in the Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram and the voucher

specimen was deposited in the Herbarium of Department of Botany, University of Kerala, Kariavattom with Accession number KUBH10169. The leaves and fruits of *F. jangomas* were collected, cleaned and shade-dried for about 3 weeks and 7 weeks respectively. Dried leaves and fruits were then homogenized to obtain coarse powder and stored in an airtight container for further use.

Preparation of extract:

Powdered leaf and fruit were extracted with methanol in the Soxhlet apparatus and the solvent extract was concentrated using a rotary vacuum evaporator and stored at 4ºC until further use. The percentage extractive yields of the methanolic extract of leaves and fruits were recorded.

Experimental animals:

Eight to ten weeks old female Sprague-Dawley (SD) rats (160-250 g) were used for acute oral toxicity. Eight to ten weeks old male Wistar rats (160-200g) were used for the hepatoprotective study. The animals were procured from Skanda Life Sciences, Bangalore. Animals were maintained as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines at a temperature of 20.2 to 22.8ºC with humidity 46-69% under 12 h dark and 12 h light cycle, and air exchanges 12-16 changes per hour. The rats were fed with a standard diet (AMRUT Laboratory Animal Feed manufactured by Pranav Agro Industries Limited, Sangli) and water ad *libitum.*

Induction of hepatotoxicity:

The paracetamol-induced hepatotoxicity model was used to study the hepatoprotective effect of *F. jangomas* leaf and fruit extract. Paracetamol was prepared in 0.25% CMC and administered orally (per os) at 640mg/kg body weight for 7 days.

Statistical analysis:

Values were expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Statistical comparisons of physicochemical and phytochemical parameters were made with Student t-test. Data from *in-vitro* antioxidant, toxicity studies and hepatoprotective studies were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests or a two-way ANOVA followed by

Bonferroni's multiple comparison test. Values for which $p<0.05$ were considered statistically significant. All the statistics have been done in GraphPad Prism 5.

BRIEF OUTLINE OF THE STUDY

The thesis is organized into four chapters:

Chapter 1: Evaluation of the physicochemical properties and phytochemicals of *Flacourtia jangomas* leaf and fruit

- Authentication of *F. jangomas* (Lour.) Raeusch plant material.
- Physicochemical, fluorescence and EDX analysis of *F. jangomas* leaf and fruit powder.
- Phytochemical analysis of methanolic extract of *F. jangomas* leaf and fruit.

Chapter 2: Evaluation of the safety of oral use of *Flacourtia jangomas* leaf and fruit methanolic extract

- Cytotoxicity effect of the *F. jangomas* leaf and fruit extract on HepG2 cells.
- Acute oral toxicity study of *F. jangomas* leaf and fruit extract on Sprague-Dawley rats.

Chapter 3: Evaluation of the hepatoprotective activity of *Flacourtia jangomas* leaf and fruit methanolic extract

- Evaluation of *in vitro* antioxidant potential of *F. jangomas* leaf and fruit extract.
- Evaluation of the hepatoprotective activity of *F. jangomas* leaf and fruit extract on paracetamol induced HepG2 cell line.
- Evaluation of the hepatoprotective activity of *F. jangomas* leaf and fruit extract on paracetamol-induced liver damage in Wistar rats.

Chapter 4: Identification of the bioactive constituents by GC-MS analysis and elucidation by molecular docking the mechanisms underlying the hepatoprotective activity of *Flacourtia jangomas*

- Identification of bioactive constituents of *F. jangomas* leaf and fruit extract using GC-MS analysis.

- Molecular docking between phytochemicals which serve as ligands and selected proteins which serve as receptors that play an essential role in different pathways in liver injury.

Chapter 1: Evaluation of the physicochemical properties and phytochemicals of *Flacourtia jangomas* leaf and fruit

1.1: Evaluation of the physicochemical properties, elemental composition and fluorescence analysis of F. jangomas

Evaluation of physicochemical parameters is important in identifying adulteration or inappropriate handling of drugs (Sudha and Srinivasan, 2013). The various physicochemical parameters such as moisture content, ash values, water and alcohol soluble extractive values, foaming index, swelling index, and pH of powdered plant material were determined by the methods described in Indian Pharmacopoeia, 1996 and WHO guidelines (Quality Control Methods for Medicinal Plant Materials, 1998). The composition of minerals was measured by energy-dispersive X-ray spectroscopy (EDX) combined with scanning electron microscopy (SEM). The powdered plant material was subjected to fluorescence analysis according to the methods of Chase and Pratt, 1949.

Ash values are used to ascertain the quality and purity of the crude drug. The water-soluble ash is used to evaluate the amount of inorganic matter and acid-insoluble ash measures the amount of silica present and it shows contamination with earthy material (Rakholiya *et al*., 2016). Results of ash values showed that the *F. jangomas* leaves and fruit are not adulterated with silicates or earthy material. The water extractive value of *F. jangomas* leaves and fruit was found to be more and better compared to the alcohol extractive value. The moisture content of *F. jangomas* leaves and fruit was found to be less, thus preventing the growth of microorganisms during storage (Rakholiya *et al*., 2016).

SEM-EDX spectra of the *F. jangomas* leaf showed a high concentration of oxygen and carbon, a moderate amount of calcium, and trace quantities of nitrogen and potassium. While the *F. jangomas* fruit showed a high concentration of oxygen and carbon and trace quantities of nitrogen and potassium. Results showed the plant is not adulterated with any heavy metals. In fluorescence analysis characteristic colour changes were observed with various chemical reagents in both the leaf and fruit of *F. jangomas* under visible and UV light (312nm) which indicate the presence or absence of different chemical constituents.

When the substances are not fluorescent by themselves, the chemical reagents promote their transformation into fluorescent derivatives (Chumbhale and Upasani, 2012).

1.2: Evaluation of the phytochemicals of F. jangomas

Phytochemicals act as the best antioxidants and protect the cells against free radical damage as well as possess different biological activity (Lobo *et al*., 2010). The *F. jangomas* extract was subjected to different qualitative and quantitative analysis to discern the bioactive components. Qualitative analysis was determined according to the Harborne method (Irum *et al*., 2019; Jothi *et al*., 2019) and quantitative analysis was measured spectrophotometrically (Jose and Anilkumar, 2018; Chandran and Indira, 2016; Roghini and Vijayalakshmi, 2018).

The preliminary phytochemical analysis of *F. jangomas* leaf and fruit has revealed the presence of various phytochemicals. Quantitative analysis of methanolic extract of *F. jangomas* leaf and fruit was found to possess flavonoids > tannins > phenols > alkaloids. Flavonoids are considered one of the most varied and prevalent groups of natural compounds. Many flavonoid compounds demonstrated to prevent injury caused by free radicals (Sudha and Srinivasan, 2013). Thus, results indicate that the plant holds high-profile values and can be used to cure various ailments caused by free radicals.

Chapter 2: Evaluation of the safety of oral use of *Flacourtia jangomas* leaf and fruit methanolic extract

2.1: Evaluation of the cytotoxic effect of the F. jangomas extract on HepG2 cells

Cell-based toxicity models are most likely to take into account the known metabolic networks at the cellular level, compared to other *in vitro* models (Madorran *et al*., 2020). The MTT system is a means of cytotoxicity testing method which measures the activity of living cells via mitochondrial dehydrogenases. Cytotoxicity effect of the *F. jangomas* leaf and fruit methanolic extract was studied in HepG2 cells using MTT Assay-[3- (4, 5 – dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt]. HepG2 cell line was procured from ATCC (ATCC® HB-8065™). Cytotoxicity studies revealed that *F. jangomas* leaf and fruit extract displayed a low level of toxicity to HepG2 cells at all the doses tested

in a dose-dependent manner. At the highest dosage (320µg/ml) tested, the extract showed less than 50% cell death.

2.2: Evaluation of toxicity study of F. jangomas on Sprague-Dawley rats

Acute toxicity studies of *F. jangomas* fruit (FJF) and leaf (FJL) extract were performed according to Organization for Economic Co-operation and Development (OECD) guidelines 423 on Sprague-Dawley rats. Eight to ten weeks old female Sprague-Dawley (SD) rats (160-250g) were used for acute oral toxicity. The animals were procured from Skanda Life Sciences, Bangalore. The study protocol was approved by the Institute Research and Ethics Committee of Skanda Life Sciences (Organization), Bangalore (Project ID number IAEC-SLS-2021-037). 50, 300, and 2000mg/kg b.wt of *F. jangomas* leaf and fruit methanolic extract was used for the toxicity study. After dosing, animals were observed individually for any abnormal behaviour and mortality for a total of 14 days. On day 15, rats were anaesthetized and blood samples were collected for the estimation of haematological (Erba transasia; H360) and biochemical parameters (Erba, Chem5X). Following blood collection, the animals were humanely sacrificed and subjected to gross necropsy. The liver, kidney, heart, and lung were collected and preserved in 10% neutral buffer formalin for histopathological examination.

The acute toxicity study of *F. jangomas* leaf and fruit extract did not show any changes in behavioural patterns and mortality. Body weight loss is frequently used as the first indicator of the adverse effect of the drugs. The dose is considered toxic if the drug causes 10 % or more body weight reduction (Rajeh *et al*., 2012). The FJF and FJL extracts did not show reduction in body weight when compared to normal control. Any toxic compounds that reach the body are exposed to blood primarily and these toxic compounds can alter the growth and development of blood components hence haematological parameters help to ascertain the toxicity by measuring the blood cells (Al-Afifi *et al*., 2018). Similarly, an increased or elevated level of these liver and kidney markers indicates hepatotoxicity and nephrotoxicity respectively (Khoo *et al*., 2018). The experimental groups did not display significant differences in haematological and biochemical parameters when compared to the control group. There were no treatment-related histopathology changes in the liver, kidney, heart, and lung in all tested rats. The histopathological results of organs confirmed the results of behavioural and biochemical parameters.

Chapter 3: Evaluation of the hepatoprotective activity of *Flacourtia jangomas* leaf and fruit methanolic extract

3.1: Evaluation of in vitro antioxidant potential of F. jangomas

The antioxidant activity of the *F. jangomas* leaf (FJL) and fruit (FJF) methanolic extracts was estimated using the DPPH radical scavenging method as described by Pant *et al*. (2015), Fe^{3+} reducing power assay by the modified method of Ngonda (2013), and the total antioxidant capacity by phosphomolybdenum assay described by Swargiary and Brahma (2017).

In DPPH radical scavenging assay, the addition of extract in a concentration-dependent manner reduces the DPPH solution (violet colour) to diphenylpicryl hydrazine (yellow colour) (Rahman *et al*., 2015). The results revealed that the *F. jangomas* leaf and fruit methanolic extract had a similar free radical scavenging activity when compared with standard ascorbic acid. Ferric reducing power assay measures the reducing potential of an antioxidant reacting with a ferric complex. The extract reduces ferric to blue-coloured ferrous (Adebiyi *et al*., 2017). The reducing power of extracts showed an increase with increasing concentration of each sample. The antioxidant capacity was measured through the phosphomolybdenum method. In this method, the extract reduces phosphomolybdate Mo (VI) to Mo (V) (green phosphate/Mo (V) compounds) (Saeed *et al*., 2012). Methanolic extract of *F. jangomas* leaf and fruit showed antioxidant activity in a dose-dependent manner. Total antioxidant capacity is a better way of depiction of the combined effect of phenolics, flavonoids and other reducing compounds in the plant extracts (Rajurkar *et al*., 2011).

3.2: Evaluation of hepatoprotective activity of F. jangomas extract on paracetamol-induced HepG2 cell line.

In vitro hepatoprotective activity of methanolic extract of *F. jangomas* leaf (FJL) and fruit (FJF) was studied against damage induced by 100µM paracetamol in HepG2 cell lines. Hepatotoxicity markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were determined using the kit method (Agappe Diagnostics). Malondialdehyde (MDA) levels were measured using lipid peroxidation assay spectrophotometrically.

The cell viability of the paracetamol control group showed a significant reduction when compared to the normal control group and this reduction in cell viability of the paracetamol control group was recovered in silymarin and *F. jangomas* leaf and fruit-treated groups which indicates the protective efficacy of *F. jangomas* leaf and fruit extract on paracetamol induced hepatotoxic HepG2 cell line. The cellular damage and loss of functional integrity of cell membranes in the liver are signs of hepatic injury (Kiran *et al*., 2012). Lipid peroxidation and free radicals are involved in the main mechanisms by which hepatotoxins injure hepatocytes (Abdel-Ghany *et al*., 2016). The elevation of AST, ALT, and LDH activities may be attributed to hepatocellular damage. There was a significant increase in AST, ALT, LDH, and MDA levels in the paracetamol control group when compared to the normal control. When compared to the paracetamol control group, silymarin, *F. jangomas* leaf and fruit could significantly reduce the activity of AST, ALT, LDH, and MDA levels. The reduced level of these enzymes in the paracetamol-induced hepatotoxicity model after methanolic extract of *F. jangomas* leaf and fruit treatment has proved its hepatoprotective effects.

3.3: Evaluation of the hepatoprotective activity of F. jangomas leaf and fruit extract on paracetamol-induced liver damage in Wistar rats.

In vivo hepatoprotective activity of methanolic extract of *F. jangomas* fruit (FJF) and leaf (FJL) was assessed in the paracetamol-induced hepatotoxic Wistar rats. Eight to ten weeks old male Wistar rats (160-200g) were procured from Skanda Life Sciences, Bangalore. The study protocol was approved by the Institute Research and Ethics Committee of Skanda Life Sciences (Organization), Bangalore (Project ID number IAEC-SLS-2021-036).

Experimental design:

Wistar rats were distributed in seven groups of six rats in each group.

G-I - Control group: Rats treated with 0.5% CMC, p.o., 7 days

G-II - Diseased control group: Rats treated with paracetamol (640mg/kg b.wt, p.o.), 7 days

G-III - Standard drug: Rats treated with paracetamol (640mg/kg b.wt, p.o.), and silymarin (100mg/kg b.wt, p.o.), 7 days

G-IV - *F. jangomas* fruit (FJF) low dose group: Rats treated with paracetamol (640mg/kg b.wt, p.o.), and FJF extract (250mg/kg b.wt, p.o.), 7 days

G-V - *F. jangomas* fruit (FJF) high dose group: Rats treated with paracetamol (640mg/kg b.wt, p.o.), and FJF extract (500mg/kg b.wt, p.o.), 7 days

G-VI - *F. jangomas* leaf (FJL) low dose group: Rats treated with paracetamol (640mg/kg b.wt, p.o.), and FJL extract (250mg/kg b.wt, p.o.), 7 days

G-VII - *F. jangomas* leaf (FJL) high dose group: Rats treated with paracetamol (640mg/kg b.wt, p.o.), and FJL extract (500mg/kg b.wt, p.o.), 7 days

The body weight of rats was observed. On day 8th, rats were anaesthetized with ketamine. Blood samples were collected for the estimation of haematological (Erba transasia; H360), biochemical parameters (Erba, Chem5X), and tumour necrosis factor-alpha (TNF-α) levels. Following blood collection, the animals were humanely sacrificed and subjected to gross necropsy. The liver was collected and preserved in 10% neutral buffer formalin for histopathological examination (Haematoxylin and Eosin staining) and the rest of the tissue was homogenized to estimate tissue parameters such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), lipid peroxidation, and tumour necrosis alpha (TNF-α). SOD and GPx were estimated using the kit method. Lipid peroxidation, CAT, and GSH were measured spectrophotometrically. TNF-α was quantified by immunoassay kits.

There was no mortality observed in rats during the 7 days of the observation period. The extract had no significant effect on the body weight of the rats when compared to the control groups. Blood, a carrier of metabolic products from and to the various regions of the cardiovascular system is affected by the clinical status of the tissue environment (Okokon *et al*., 2016). The results showed that treatment with paracetamol induces changes in haematological parameters in rats. White blood cell (WBC) count was significantly increased and platelet, haemoglobin, red blood cells (RBC) and packed cell volume (PCV) levels were significantly decreased in the paracetamol-intoxicated group when compared to the normal control. Administration of silymarin, FJF (250 and 500mg/kg) and FJL (250 and 500mg/kg) significantly decreased the WBC count and significantly increased haemoglobin levels compared to the paracetamol-intoxicated group. Administration of silymarin and FJL (250 and 500mg/kg) significantly increased platelet, RBC and PCV levels compared to the paracetamol-intoxicated group.

Decreased levels of total protein and albumin were observed in the paracetamol-intoxicated group. Administration of silymarin, FJF (500mg/kg) and FJL (250 and 500mg/kg) significantly restored the total protein and administration of silymarin, FJF (500mg/kg) and FJL (500mg/kg) significantly restored the albumin levels. Restoration of albumin and protein levels suggests the stabilization of the endoplasmic reticulum leading to protein synthesis and hepatocyte regeneration (Matic *et al*., 2016). An increased level of bilirubin was observed in the paracetamol-intoxicated group suggesting excessive heme destruction and blockage of the biliary tract (Kanchana and Sadiq, 2011). Administration of silymarin, FJF (500mg/kg) and FJL (250 and 500mg/kg) were able to prevent the increase in the serum level of total bilirubin suggesting liver protection. Increased level of aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) was observed in the paracetamol-intoxicated group indicating cellular leakage and loss of functional integrity of the cell membrane (Iroanya, 2014). Administration of silymarin, FJF (500mg/kg) and FJL (250 and 500mg/kg) were able to significantly decrease AST, ALT, and ALP levels compared to the paracetamol-intoxicated group suggesting stabilization of the plasma membrane. A significant increase in serum creatinine, urea and uric acid levels observed in the paracetamol-intoxicated group may be indicators of acute tubular necrosis caused by paracetamol toxicity indicating nephrotoxicity (Canayakin *et al*., 2016). Administration of silymarin, FJF (500mg/kg) and FJL (250 and 500mg/kg) were able to significantly restore the serum creatinine and uric acid levels and administration of silymarin, FJF (250 and 500mg/kg) and FJL (250 and 500mg/kg) were able to significantly restore the urea levels indicating its nephroprotective nature.

A significant increase in total cholesterol, triglycerides, low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) levels and a decrease in high-density lipoprotein (HDL) level was observed in the paracetamol-intoxicated group suggesting the altered membrane structure and function as well as lipids metabolism in the liver (Okokon *et al*., 2016). Administration of silymarin, FJF (500mg/kg b.wt) and FJL (250 and 500mg/kg) extract significantly restored the level of total cholesterol, triglycerides, HDL, LDL and VLDL to near normal which indicates that our extracts have a beneficial effect on lipid profile by lifting good cholesterol (HDL) and dropping-down bad cholesterol (LDL). Significant elevation in serum and hepatic tissue TNF-α levels was observed in the paracetamol-intoxicated group. Administration of silymarin, FJF (500mg/kg b.wt) and FJL (500mg/kg b.wt) induced a significant reduction in the elevated serum TNF-α levels and

administration of silymarin, FJF (250 and 500mg/kg) and FJL (250 and 500mg/kg) induced a significant reduction in the tissue level of TNF-α. This improvement is a definite indication of the direct antioxidant potential of FJF and FJL.

Significant depletion in SOD, CAT, GSH and GPx levels was observed in the paracetamol-intoxicated group. A clear manifestation of inactivation or failure of the antioxidant enzymes to scavenge the excessive formation of free radicals resulting in the accumulation of free radicals and redox imbalance which in turn propagates oxidative stress and liver injury (Ali, 2012). Administration of silymarin, FJF (250 and 500mg/kg) and FJL (250 and 500mg/kg) were able to significantly increase the antioxidant enzymes thereby decreasing the free radical generation and protecting the liver from damage induced by paracetamol intoxication. Polyunsaturated fatty acid and methylene groups in the phospholipids of cell membranes are very sensitive to oxidation by radicals hence more prone to lipid peroxidation. Malondialdehyde (MDA), is the end product of lipid peroxidation which alters the functions of the cell by binding with DNA and proteins (Ali, 2012). A significant increase in MDA levels was observed in the paracetamol-intoxicated group and has reverted to normal levels in the silymarin, FJF (250 and 500mg/kg) and FJL (250 and 500mg/kg) treated groups. Therefore, the hepatoprotective activity of the tested *F. jangomas* fruit and *F. jangomas* leaf extracts may be attributed to their antioxidant potential.

The results of the histopathological examination of the liver supported the biochemical investigations. The liver section of Group I (normal control rat group) showed a normal central vein, and portal vein with radially arranged hepatocytes. Group II (paracetamol intoxicated group) showed loss of normal architecture of hepatocytes with swollen hepatocytes with vacuolated cytoplasm and rounded vesicular nuclei, showing a focal area of micro abscess consisting of inflammatory cells and necrotic debris, some nuclei are darkly stained - nuclear pyknosis. Group III (silymarin-treated group) showed a normal central vein with hepatocytes. Group IV and VI (FJF and FJL (250mg/kg b.wt) treated groups) showed normal central veins with mild vacuolations and ballooned hepatocytes. Group V and VII (FJF and FJL (500mg/kg b.wt) treated groups) showed a normal central vein with hepatocytes.

Chapter 4: Identification of the bioactive constituents by GC-MS analysis and elucidation by molecular docking the mechanisms underlying the hepatoprotective activity of *F. jangomas*

4.1: Identification of bioactive constituents of F. jangomas leaf and fruit extract using GC-MS analysis.

The phytochemical investigation of the methanolic extract of *F. jangomas* leaf and fruit was performed on Shimadzu GC-MS (Model Number: QP2010S) instrument. The results were compared by using the Wiley Spectral Library search program (Wiley 8) and NIST mass spectral database (NIST 11).

The spectrum profile of GC-MS analysis confirmed the presence of nineteen major constituents in the methanolic extract of *F. jangomas* leaves which includes benzoic acid, 2,3-dihydro-benzofuran, decanal, megastigmatrienone, 4-nitro-5-hydroxy-1,2-dimethylindole, chinasaure, blumenol, calendin, phytol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, hexadecanoic acid, methyl octadeca-9,12-dienoate, linolenic acid, 2-octyl benzoate, stigmast-5-en-3-ol, (3.beta.)-, lupeol, squalene, and vitamin E and seven major constituents in the methanolic extract of *F. jangomas* fruits which includes 4H-Pyran-4-One, 2,3-Dihydro-3,5-Dihydroxy-6-Methyl-, 5-Hydroxymethylfurfural, Quinic acid, Chinasaure, Methyl Palmitate, 9,12-Octadecadienoic acid, and 9-Octadecenoic Acid (Z)-.

4.2: Molecular docking between phytochemicals which serve as ligands and selected proteins which serve as receptors that play an essential role in different pathways in liver injury.

Compounds obtained from GC-MS analysis of *F. jangomas* leaf and fruit were docked against the proteins involved in hepatotoxicity and oxidative stress. 3D structures of bioactive compounds were collected from Pubchem, a compound database (Based on the availability of the structure of ligands in Pubchem). Some phytochemical compounds with their PubChem ID are listed below. Benzoic Acid (243), 2,3-Dihydro-Benzofuran (10329), Decanal (8175), Megastigmatrienone (5375190), 4-Nitro-5-Hydroxy-1,2-Dimethylindole (609757), Chinasaure (6508), Blumenol (5280462), Phytol (6428538), 3,7,11,15-Tetramethyl-2-Hexadecen-1-Ol (5366244), Hexadecanoic Acid Methyl Ester (985), Methyl Octadeca-9,12-Dienoate (8203), Linolenic Acid Methyl Ester (5280934), 2-Octyl Benzoate

(243800), Lupeol (259846), Squalene (638072), Vitamin E (14985) and 4H-Pyran-4-One,2,3-Dihydro-3,5-Dihydroxy-6-Methyl- (119838), 5-Hydroxymethylfurfural (237332), Chinasaure (6508), Methyl Palmitate (8181), 9,12-Octadecadienoic acid methyl ester (3931), 9-Octadecenoic Acid (Z)- Methyl Ester (965) respectively. These compounds were used in the docking experiment. The protein structure was retrieved from a protein data bank (http://www. rcsb.org) with PDB ID: 1ILG (Human Pregnane X Receptor), 1N3U (oxygenase-1), 2LV3 (GSH reductase), 1NFI (IkappaBalpha/NF-kappaB complex), 3E6I (cytochrome P450 2E1), 1XU2 (TNF receptor) (Bulbul *et al*., 2020, Galani *et al*., 2020, Dutta *et al*., 2018).

Upon a series of receptor-ligand interaction studies, it was identified that each of the ligands has a different binding affinity with the selected proteins. The free energy of binding (ΔG) can be related to the binding affinity i.e., the lower the value higher the ligand affinity to the active site of the target protein. It is seen that Lupeol has the highest interaction with all the receptors on average followed by Vitamin E and 4-Nitro-5-Hydroxy-1,2-Dimethylindole. For the comparative analysis, silymarin was used as a standard. Silymarin is a proven drug against hepatotoxicity. Lupeol had a binding affinity similar to silymarin with all the receptors on average.

CONCLUSION

Liver diseases are the most common health problem in the global scenario and it is mainly due to their function as the major site for drug metabolism. However, many drugs are known to cause liver injury. Conventional and synthetic drugs available now to treat liver diseases are sometimes inadequate and can cause serious adverse effects.

Naturally occurring antioxidants include flavonoids, tannins, phenols, and lignans. The methanolic extract of *F. jangomas* showed a good range of bioactive phytoconstituents such as phenol, flavonoid, tannin, and alkaloid contents. Most of the drug-induced toxicity is linked with oxidative stress as a result various therapy for the treatment of liver injury now available is mainly involved with an antioxidant defence system. Hence *F. jangomas* leaf and fruit methanolic extract may contribute to treating hepatotoxicity induced by paracetamol-induced oxidative stress-related liver injury. *In vitro* hepatoprotective activity of methanolic extract of *F. jangomas* leaf and fruit showed

protection for the hepatocyte by inhibiting lipid peroxidation and stabilizing the hepatocellular membrane.

All the haematological, biochemical, lipid profile and antioxidant findings suggest that the changes in these parameters in the paracetamol control group were reversed in the treated groups. The fruit extract treated group (250mg/kg b.wt) showed less response in all the parameters compared to the other treated groups. However, all the treated groups showed good hepatoprotection when compared to the disease control group and moderate hepatoprotection compared to the positive control group i.e., the silymarin-treated group. The results obtained were further supported by the histopathological studies on the liver tissue which suggests that *F. jangomas* plays an important role in maintaining the structural integrity of the hepatocellular membrane. The active constituents in both the *F. jangomas* leaf and fruit extracts were identified by GC-MS analysis and revealed that the leaves and the fruit are a rich source of phytoconstituents. Lupeol had a binding affinity similar to silymarin with all the receptors on average.

Antioxidant phytochemicals of *F. jangomas* may be responsible for the observed hepatoprotective activity and the active metabolites present in extract gives nutrition to the hepatocytes. Hence, overall results indicated methanolic extract of *F. jangomas* leaf and fruit possess a high degree of protection against the hepatotoxic effect of paracetamol.

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PAPER PUBLICATION:

- Pai, A., & Shenoy, C.K. (2021). Physicochemical and phytochemical analysis of methanolic extract of leaves and fruits of *Flacourtia jangomas* (Lour.) Raeusch. *International Journal of Pharmaceutical Sciences and Research,* 12(3): 1671-1678. E-ISSN: 0975-8232; P-ISSN: 2320-5148; Web of Science, Cited by 2

- Pai, A., & Shenoy, C.K. (2021). Hepatoprotective activity of *Flacourtia jangomas* (Lour.) Raeusch leaves and fruit methanolic extract on paracetamol-induced hepatotoxicity in HEPG2 cells. *Biomedicine.* 41(3): 587-591. P-ISSN: 0970-2067; https://www.scopus.com/sourceid/28618.

- Pai, A., & Shenoy, C.K. (2023). Toxicity and safety profiling of *Flacourtia jangomas* (Lour.) Raeusch fruit and leaf methanolic extract in Sprague Dawley rats. *Journal of Applied Biology and Biotechnology,* ISSN/EISSN: 2455-7005/2347-212X (Accepted for publication); https://www.scopus.com/sourceid/21100970232.

PAPER PRESENTATION:

- Presented a research paper on "Phytochemical and antioxidant studies on methanolic extract of fruit of *Flacourtia jangomas* (Lour.) Raeusch" at the International Conference on Contriving Therapeutic Drugs from Medicinal Plants (CTDMP-2020) at the Department of Biochemistry, Sree Narayana Guru College, Coimbatore on12th February 2020.

- Presented a research paper on "Phytochemical and antioxidant studies on methanolic extract of leaf of *Flacourtia jangomas* (Lour.) Raeusch" in Alumni sponsored one-day National Conference on Current Advances in Medical and Industrial Biotechnology at Department of Biotechnology, Alva's College, Moodubidire on 20th February 2020.

- Presented a research paper on "Antioxidant potential of *Flacourtia jangomas* (Lour.) Raeusch leaf extract on paracetamol induced hepatic injury" at the International Conference on Recent Advances in Biological Sciences 2023 organised by Inter-University Centre for Evolutionary and Integrate Biology, University of Kerala, Kariavattom, Thiruvananthapuram on 17th to 19th January 2023.

- Presented a research paper on “Phytochemical screening and antioxidant potential of *Flacourtia jangomas* (Lour.) Raeusch fruit extract on paracetamol induced hepatic injury” at the International Conference cum Workshop on Plant Molecular Biology and Bioinformatics, Department of Bioinformatics, Pondicherry University, Indian Science Congress Association (ISCA), Pondicherry Chapter and Society for Plant Research (SPR), Pondicherry on 13th to 15th February 2023.

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